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Biological and molecular characteristics of *Beauveria bassiana* isolates from California *Lygus hesperus* (Hemiptera: Miridae) populations

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Abstract

Lygus hesperus is an important pest of many crops grown in the Western US. In addition, other species of *Lygus* cause damage in other parts of the world. To date, no selective pesticide exists for the control of *Lygus* spp. and broad spectrum pesticides that also kill natural enemies may lead to secondary pests. Entomopathogenic fungi may offer an alternative to chemical pesticides. Isolates of *Beauveria bassiana* collected from San Joaquin Valley of California (SJV) *L. hesperus* populations were screened for their ability to grow at high temperatures and for their ability to infect and kill *L. hesperus* adults and nymphs under laboratory conditions. No isolate grew at 37 or 35 °C but most isolates were able to grow at 32 °C. In addition, one *L. hesperus* isolate was more efficacious at higher doses than the commercial isolate. Microsatellite markers were used to determine that selected isolates could be distinguished from other isolates. Preliminary information suggested 82 SJV isolates of *B. bassiana* were closely related to each other but distantly related to the commercial isolate.
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Keywords: Entomopathogenic fungi; Microbial pesticide; Western tarnished plant bug; Microsatellite markers; SSR

1. Introduction

Lygus hesperus Knight (Heteroptera: Miridae) is an important insect pest of many crops across the Western US and Canada. Feeding by nymphs and adults on reproductive plant parts results in direct economic damage to several crops including strawberries, cotton, and seed alfalfa. Control of *L. hesperus* is typically done with synthetic chemical pesticides applied according to scouting guidelines. However, there are no pesticides that are selective for *L. hesperus* and arthropod natural enemies also suffer significant mortality. In an effort to identify the extent of naturally occurring parasites and pathogens of *L. hesperus*, McGuire (2002) reported widespread, and at times, high prevalence of the fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin throughout the San Joaquin Valley (SJV) of California. No parasites were recovered in that study though efforts are now underway to introduce the parasitoid *Peristenus digoneutis* into *L. hesperus* populations (Woods, 2004.).

The prevalence of *B. bassiana* adapted to the hot dry climate of the SJV is stimulus for development of a microbial pesticide that may impact *L. hesperus*. Several commercial products based on *B. bassiana* have been available in California for control of aphids and whiteflies but none have achieved widespread use. Attempts to control *L. hesperus* and *Lygus lineolaris* under field conditions with Mycotrol (Emerald BioAgriculture, Lansing, MI) have been inconsistent (Noma and Strickler, 1999; Steinkraus and Tugwell, 1997) despite reasonably good laboratory results. Mycotrol is a product based on

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a *B. bassiana* strain that may not be particularly adapted to a Heteropteran host or a hot dry environment. Indeed, Steinkraus and Tugwell (1997) reported significantly higher field activity with a *B. bassiana* strain isolated from an Arkansas *L. lineolaris* population than with Mycotrol. These data support the hypothesis presented by Fargues and Remaudiere (1977) that strains of entomopathogenic fungi isolated from the target host and environment will be more effective than other strains of the same species.

As a first step toward identifying isolates of *B. bassiana* for further development and field testing, we measured the efficacy of selected isolates against *L. hesperus* and the growth rate of selected isolates at high temperatures. In addition, 82 isolates from the SJV were genetically characterized with seven microsatellite markers or single sequence repeats (SSR) described by Rehner and Buckley (2003). The technology enables very detailed discrimination among isolates and may be used to address questions of population structure and genetic relatedness.

2. Materials and methods

2.1. Isolation and culture of B. bassiana

During routine sampling for prevalence and distribution of *B. bassiana* in *L. hesperus* populations, insects that died from mycosis and sporulated were placed on agar selective for *B. bassiana* and *Metarhizium* spp. (Doberski and Tribe, 1980) (see Table 1 for a listing of isolates). Once a colony was established from an individual insect, the fungus was transferred to Sabouraud's dextrose agar (Becton–Dickson, Cockeyesville, MD) supplemented with 1% yeast extract (Sigma, St. Louis, MO) (SDAY) and incubated at 28 °C. A subset of these collections has been deposited in the USDA-ARS Entompathogenic Fungal Culture Collection (ARSEF), Ithaca, NY. Conidia were harvested from culture plates after 10–14 days incubation by scraping with a sterile rubber policeman into 0.01% Silwet L-77 (GE Silicones, Friendly, WV). The conidia were enumerated and viability determined by adding a sample of approximately 10^7 conidia to 20 ml potato dextrose broth (Sigma) and incubating ca. 16 h in a rotary shaker at 28 °C. Conidia germination was examined under a compound microscope at 400× and scored viable if the germ tube was at least twice the length of the conidium. Percentage viability was measured on 250 conidia. For preservation, glycerol (qs 10% v/v) was added to the conidial suspension and stored in aliquots of 2×10^8 in 2 ml solution at −80 °C until needed for assays. All bioassays were conducted on the basis of viable conidia measured after thawing. The strain from Mycotrol (GHA) was isolated and cultured as above to eliminate effects of production

Table 1
Beauveria bassiana isolates collected from San Joaquin Valley, CA populations of *L. hesperus*

Designation	Field	Isolate	Year collected	Julian day	County (CA)
01-25	1	25	2000	326	Kern
02-40	2	40	2000	326	Kern
02-44	2	44	2000	326	Kern
02-48	2	48	2000	326	Kern
02-51	2	51	2000	326	Kern
02-65	2	65	2000	326	Kern
02-75	2	75	2000	326	Kern
02-85	2	85	2000	326	Kern
02-93	2	93	2000	326	Kern
03-3	3	3	2000	326	Kern
03-4	3	4	2000	326	Kern
03-5	3	5	2000	326	Kern
03-6	3	6	2000	326	Kern
03-9	3	9	2000	326	Kern
03-10	3	10	2000	326	Kern
03-12	3	12	2000	326	Kern
03-13	3	13	2000	326	Kern
03-16	3	16	2000	326	Kern
03-24	3	24	2000	326	Kern
03-29	3	29	2000	326	Kern
03-30	3	30	2000	326	Kern
03-33	3	33	2000	326	Kern
03-36	3	36	2000	326	Kern
03-43	3	43	2000	326	Kern
03-44	3	44	2000	326	Kern
03-48	3	48	2000	326	Kern
03-49	3	49	2000	326	Kern
03-50	3	50	2000	326	Kern
03-51	3	51	2000	326	Kern
03-58	3	58	2000	326	Kern
03-59	3	59	2000	326	Kern
03-60	3	60	2000	326	Kern
03-61	3	61	2000	326	Kern
03-63	3	63	2000	326	Kern
03-64	3	64	2000	326	Kern
03-65	3	65	2000	326	Kern
03-66	3	66	2000	326	Kern
03-67	3	67	2000	326	Kern
03-68	3	68	2000	326	Kern
03-70	3	70	2000	326	Kern
03-81	3	81	2000	326	Kern
03-82	3	82	2000	326	Kern
03-96	3	96	2000	326	Kern
03-100	3	100	2000	326	Kern
04-11	4	11	2000	326	Kern
04-15	4	15	2000	326	Kern
04-43	4	43	2000	326	Kern
04-49	4	49	2000	326	Kern
04-62	4	62	2000	326	Kern
04-93	4	93	2000	326	Kern
05-40	5	40	2000	326	Kern
05-47	5	47	2000	326	Kern
05-57	5	57	2000	326	Kern
05-61	5	61	2000	326	Kern
05-70	5	70	2000	326	Kern
05-84	5	84	2000	326	Kern
17-27	17	27	2001	179	Tulare
17-41	17	41	2001	179	Tulare
18-05	18	5	2001	179	Tulare
22-19	22	19	2001	184	Fresno
35-26	35	26	2001	186	Madera
38-05	38	05	2001	212	Madera

Table 1 (*continued*)

Designation	Field	Isolate	Year collected	Julian day	County (CA)
38-06	38	06	2001	212	Madera
38-12	38	12	2001	212	Madera
38-34	38	34	2001	212	Madera
44-02	44	2	2001	183	Kern
44-03	44	3	2001	219	Kern
44-39	44	39	2001	214	Kern
44-42	44	42	2001	219	Kern
47-41	47	41	2001	214	Merced
48-28	48	28	2001	183	Kern
48-47	48	47	2001	212	Kern
48-49	48	49	2001	212	Kern
49-59	49	59	2001	212	Kern
54-12	54	12	2001	163	Kern
54-13	54	13	2001	219	Kern
54-29	54	29	2001	219	Kern
54-38	54	38	2001	219	Kern
54-43	54	43	2001	219	Kern
54-45	54	45	2001	219	Kern
54-47	54	47	2001	219	Kern
56-06	56	6	2001	177	Kern
56-35	56	35	2001	219	Kern
56-44	56	44	2001	212	Kern
GHA[a]	N/A	N/A	2003		

[a] GHA is the designation for the isolate within the commercial product Mycotrol (Emerald BioAgriculture).

methods and formulation ingredients on insecticidal activity. Glycerol was not removed prior to using the conidia in assays.

2.2. Temperature tolerance

Isolates were selected for testing based on the Julian date of isolation to more closely focus the efforts. Cultures were established by spreading 1 ml of 0.01% Silwet L-77 containing 10^6 conidia onto a 9 cm diameter petri dish containing SDAY. The culture was allowed to grow for approximately 3-days at 28 °C or until an even fungal mat was visible across the entire dish. A flamed 9 mm diameter brass cork borer was then used to transfer a plug of the culture including media onto the surface of a 9 cm diameter petri dish containing 20 ml SDAY. All plates were wrapped twice with stretched Parafilm (Pechiney Plastic Packaging, Menasha, WI). Four plates were established for each isolate for each temperature and placed inverted in dark incubators at 28, 32, 35, or 37 °C (all ±1 °C as measured by HOBO temperature recorders (Onset, Pocasset, MA) placed in each chamber). Radial growth was measured every 2–3 days for 24 days by averaging the diameter of the colony on two axes with a caliper. Data were analyzed across all isolates by ANOVA and a protected least significant difference was calculated (Statistix, 1997) to determine significant growth differences among isolates within a given temperature. Because isolates did not grow at 35 or 37 °C and differences in growth rates at 28 °C were not statistically significant ($F = 1.49$, df = 12, 39; $P = 0.17$), the assay was repeated only at 32 °C in an incubator used for another temperature in the previous test, to confirm results. Because there was no significant date by isolate interaction, the two assays were combined and a least significant difference was calculated.

2.3. Activity against L. hesperus

Lygus hesperus was reared in the laboratory on a diet of fresh, washed green beans and raw sunflower seeds. Adults were allowed to oviposit on green beans for two days, then the beans were moved to 4 L plastic, ventilated tubs for nymph emergence and development. Nymph tubs were supplemented with additional green beans every 2–3 days. Shredded paper was placed in all cages to allow separation of individuals. Adults were collected from alfalfa fields approximately three times each year, held separately for one generation and then their offspring were added to the colony to avoid genetic bottlenecks. Periodic microscopic investigations determined the colony to be free from microsporidia infections. The colony room was maintained at 28 ± 3 °C and 50–60% relative humidity with a 16L:8D photoperiod. Adults that had emerged over a two day period were used in most experiments. Nymphs in the 3rd–4th instar from a single egg cohort were used in the other assays.

A specially constructed spray chamber was used to spray conidia directly onto insects. The chamber consisted of a 31 cm diameter Plexiglas cylinder that was placed over a revolving 30 cm diameter aluminum turntable with a Plexiglas template that allowed placement of a 9 cm diameter petri plate in the center of the turntable. The top of the Plexiglas cylinder was fitted with a Plexiglas disk that had a port for the spray apparatus. The spray apparatus consisted of a TG04 Full Cone spray nozzle (Spraying Systems, Wheaton, IL) with a 45 ml test tube containing the sample. Positive pressure (approximately 1546 g/cm^2 (22 psi)) was introduced into the test tube with compressed air thus expelling the sample through the nozzle onto the petri dish rotating at the bottom of the chamber. The distance between nozzle and dish was 60 cm and allowed for full coverage of the turntable. The entire spray chamber was enclosed within a Plexiglas box (90 high × 45 × 45 cm) with a hepa-filtered vent. Each spray sample was comprised of 5 ml of 0.01% Silwet-L77 in DI water containing conidia. The spray tower was calibrated by placing four 18 × 18 mm glass coverslips in the bottom of eight petri dishes each receiving one of four doses of conidia to determine spore deposition. All four coverslips were pooled and carefully washed into 0.01% Silwet-L and the conidia were enumerated. Microscopic evaluation of coverslips after washing revealed very few conidia (<200) remaining after the washing procedure. A paired *t* test revealed no significant difference between observed conidial deposi-

tion and that expected from simple calculations of spray volume and conidial concentration.

Controls consisted of 0.01% Silwet-L77 only. Conidia were collected and quantified from *B. bassiana* cultures on SDAY as previously described and diluted to 10^4, 10^5, 10^6 or 10^7 per ml in 0.01% Silwet L-77. All doses applied were based on viable conidia quantified the day before each assay. The 10^7 per ml dose deposits approximately 7×10^4 spores/cm^2. Twenty adults, lightly anesthetized with CO_2 were used for each dose and after treatment adults were transferred to individual five dram plastic vials (Thornton Plastics, Salt Lake City, Utah) containing pieces of fresh green bean. Vials were capped with ventilated lids and held at 28 °C with a 16L:8D photoperiod for 7 days. Insects were checked for mortality and beans were changed every 2–3 days. Dead insects were transferred to 1% water–agar and held at 28 °C for at least 3 days to determine if the fungus survived in the insect to sporulation. Eight assays were conducted on different days to examine 14 different isolates including GHA. Based on those results, single spore colonies were derived from isolates 17-41, 44-03, 54-43, and GHA and additional assays were then done to determine reproducibility of results. In each assay, GHA was tested as a standard so each new isolate could be compared for relative efficacy. Isolates 17-41 and 54-43 were each tested twice more and isolate 44-03 was tested three additional times. Finally, three assays were done to compare efficacy of one isolate (44-03) with GHA against nymphs. Due to a limited supply, only 10 nymphs per dose were used in each nymphal assay. The LC_{50}s were calculated for the initial eight assays, after correcting for control mortality (Abbott, 1925), using a GWBASIC probit model based on Finney (1971). Doses that resulted in visible sporulation on the cadaver in 50% of the treated population (SC_{50}) were calculated without correction. Due to significant χ^2 for heterogeneity from several probit calculations, the data from the repeated adult assays and three nymph assays were handled differently. Corrected percentage mortality and sporulation were arcsine transformed and data were analyzed using ANOVA (Statistix). If mortality or sporulation was significantly different among isolates, pairwise comparisons were made between GHA and the SJV isolates at a common dose using a *t* test.

2.4. Genetic characterization

Fungal cultures were grown on SDAY for 5 days and prepared for DNA analysis. Approximately 50 mg of mycelia were collected from each isolate, and total genomic DNA was extracted with Dneasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

Seven PCR primer pairs (Table 2) previously developed (Rehner and Buckley, 2003), which flank simple sequence repeats (SSR) were used to screen the *B. bassiana* isolates. PCR primers were synthesized by Proligo (Boulder, CO).

Table 2
PCR-based microsatellite (SSR) markers used to determine the genetic relatedness of *B. bassiana* isolates

Locus (GenBank Accession No.)	Number of alleles	Size range of alleles (bp)
Ba01 (AY212020)	7	84–105
Ba02 (AY212021)	6	123–144
Ba03 (AY212022)	9	124–164
Ba05 (AY212023)	7	113–187
Ba06 (AY212024)	3	104–117
Ba08 (AY212025)	4	199–284
Ba13 (AY212027)	3	151–201

Locus information, repeat motif, and primer sequences (F, forward; R, reverse) can be found in Rehner and Buckley (2003). The number of alleles and their size range observed from 82 isolates are shown.

PCR amplification was performed in a total volume of 20 μl containing 20 ng of template DNA, 0.1 μM of each primer (forward and reverse), 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U *Taq* polymerase (Amplitaq, Applied Biosystem, Foster city, CA) with cycling profile of 1 cycle of 2 m at 94 °C, 10 cycles of 15 s at 94 °C, 30 s at 60 °C (step −0.5 °C/cycle for cycles 2–10), and 1 m at 72 °C, 35 cycles of 15 s at 94 °C, 30 s at 55 °C, 1 m at 72 °C, and a final extension of 6 m at 72 °C. PCR products were first checked and separated on a 3% Super Fine Resolution (SFR) agarose (Amresco, Solon, OH) gel containing 1× TBE at 80 V for 4–5 h, and visualized by Alpha imager, V5.5 (Alpha Innotech, San Leandro, CA) after staining with ethidium bromide. After primer pairs were amplified and resulted in discrete PCR banding patterns, primers were again synthesized and labeled with RED Well Dye D2, D3, D4 for their use with a CEQ 8000 DNA Analysis System (Beckman Coulter). The seven primer pairs amplified DNA fragments from a SSR locus in all isolates examined. Each allele was designated by locus (based on the associated primer pair) followed by a number 1–7, which corresponded to its molecular weight where 1 is the highest and 7 is the lowest.

To determine the genetic similarity among the isolates, pair-wise genetic similarity coefficients were calculated based on Jaccard's similarity coefficient (Jaccard, 1908) to evaluate the pattern of genetic variation among isolates. A similarity coefficient was calculated as $[a/(n-d)]$ for each pair of isolates, where a is the number of alleles for which a band-peak is present and d is the number of alleles for which the band-peak is absent, and n is the total number of alleles. A dendrogram was constructed using the unweighted pair group method average (UPGMA) clustering analysis of the genetic similarity coefficient matrices. Secondary analyses were performed using PAUP*4b.10 software (Swofford, 1998) to compare results generated by different methods such as Jaccard's similarity coefficient, the neighboring joining (N-J) method (Saitou and Nei, 1987), and bootstrapping (Felsenstein, 1985).

3. Results and discussion

3.1. Radial growth

All isolates tested grew at 28 °C (Table 3) and no significant differences in growth rate were observed ($F = 1.49$, df = 12, 39; $P = 0.17$). No isolates grew at 35 or 37 °C and isolates grew at varying rates at 32 °C. Many of the isolates grew significantly faster at 32 °C than the commercial GHA isolate. Interestingly, although all the isolates under investigation were obtained in the SJV in the heat of the summer, not all of them grew faster than GHA. In fact 56-06 did not grow at all at 32 °C. After 24 days, isolates from the 35 and 37 °C incubators were moved to 28 °C. All isolates from the 35 °C incubator were able to resume growth but none of the isolates exposed to 37 °C for 24 days grew. Further tests should reveal if the isolates can withstand shorter exposures to 37 °C. Inglis et al. (1996) showed that when GHA was exposed to relatively short times of exposure to higher temperatures, the fungus did not grow, but could still cause mortality of grasshoppers when the temperature was reduced to optimal ranges. Mass production may also be affected at higher temperature, possibly by shortening the production time necessary to achieve conidia. Studies are in progress to assess the ability of these isolates to produce conidia in economic quantities under semi-commercial conditions. Ability to grow at high temperatures may be particularly important because infection processes and growth of the fungus within the hemocoel is affected by temperature. Liu et al. (2003) identified isolates from *L. lineolaris* that would grow at 35 °C and suggested this was one important attribute in selecting isolates for further development.

Table 3
Growth rate (mm/day) of *B. bassiana* isolated from *L. hesperus* in the San Joaquin Valley, California at 28 and 32 °C (see Table 1 for isolate details)

Isolate	28 °C	32 °C
05-59	1.94 (2.32)	1.57 (2.93)
17-41	1.20 (0.23)	1.49 (0.61)
35-26	1.40 (0.26)	0.92 (0.61)
38-05	1.67 (1.20)	1.46 (1.06)
38-06	2.09 (0.30)	1.76 (2.11)
44-41	0.92 (0.15)	0.88 (0.23)
54-38	1.56 (0.80)	ND[a]
54-43	1.74 (1.13)	1.19 (0.21)
54-45	1.56 (0.78)	1.37 (1.64)
56-06	1.50 (0.31)	0.00 (0.00)
56-35	1.41 (0.53)	ND
GHA	0.91 (1.08)	0.84 (0.18)
44-03	1.33 (2.63)	0.74 (0.35)
F (df)	1.49 (12, 39)	21.35 (10, 63)
CVC[b]	NS	0.31

[a] Not determined.
[b] Critical value for comparison ($P < 0.05$).

3.2. Bioassay

LC_{50}s for mortality and SC_{50}s for sporulation were calculated to identify any differences among strains with

Table 4
Effect of *B. bassiana* isolates on laboratory reared adult *L. hesperus*

Assay No. (percentage control mortality)	Isolate	Mortality					Sporulation				
		LC_{50} ($\times 10^5$)[a]	UCL ($\times 10^5$)	LCL ($\times 10^5$)	χ^2	Slope (SE)	SC_{50} ($\times 10^5$)[b]	UCL ($\times 10^5$)	LCL ($\times 10^5$)	χ^2	Slope SE)
1 (11)	GHA	1.1	2.2	0.49	2.88	1.23 (0.24)	1.8	3.5	0.92	0.68	1.45 (0.27)
	56-35	1.9	4.3	0.77	2.84	0.97 (0.18)	4.3	10	1.9	2.45	0.98 (0.18)
2 (0)	54-45	3.2			7.9	0.60	1.2	46.0	0.24	2.5	0.42 (0.14)
	49-59	1.3	3.5	0.61	4.4	1.05 (0.21)	3.6	16.0	1.4	3.8	0.88 (0.20)
3 (0)	44-03	0.058	0.23	0.0012	0.07	0.7 (0.2)	0.058	0.23	0.0012	0.07	0.70 (0.20)
	38-06	1.1			10.6	0.88 (0.17)	1.8			15.2	0.92 (0.17)
4 (5)	54-43	0.34	0.85	0.086	0.03	0.90 (0.20)	0.33	1.0	0.042	0.04	0.67 (0.17)
	GHA	0.36	0.83	0.12	0.34	1.01 (0.17)	0.7	1.6	0.25	0.18	0.98 (0.19)
	38-05	0.47	1.3	0.11	0.98	0.79 (0.17)	1.3	3.7	0.39	0.92	0.72 (0.15)
5 (10)	54-38	1.3			7.5	1.3 (0.22)	1.0	2.2	0.5	6.8	1.2 (0.12)
6 (0)	GHA	0.28	0.76	0.058	3.95	0.79 (0.18)	0.87	2.2	0.26	5.0	0.80 (0.16)
	17-41	0.17	0.65	0.0068	1.4	0.60 (0.17)	0.28	0.91	0.028	1.8	0.66 (0.17)
7 (0)	56-06	0.69			125.1	2.0 (0.41)	0.86			7.5	1.7 (0.33)
	35-26	0.27			7.4	0.49 (0.15)	0.038	0.24	0.0003	5.4	0.52 (0.17)
8 (6)	54-13	0.41	1.6	0.023	6.3	0.50 (0.14)	3.7	12.0	1.2	6.6	0.68 (0.15)
	47-41	0.14	0.81	0.0002	3.8	0.42 (0.14)	9.9	77	2.5	3.5	0.51 (0.14)
	44-03	1.1	4.0	0.22	0.38	0.93 (0.26)	7.7	90.6	1.4	0.45	0.66 (0.21)

Blank CL values indicate significant χ^2 goodness of fit making calculations unreliable.
[a] Conidia/ml required to kill 50% of the test population after Abbott's correction for control mortality.
[b] Conidia/ml required for 50% of the test population to sporulate.

Table 5
Percentage mortality and sporulation of *L. hesperus* in response to selected isolates of *B. bassiana*

Conidia per ml	Average percentage mortality (SE) (adults)			Average percentage sporulation (SE) (adults)		
	GHA	*t*	54-43	GHA	*t*	54-43
10^4	13.3 (4.7)		25.7 (0.7)	6.7 (2.4)		25.7 (2.4)
10^5	34.7 (13.9)		30.1 (22.9)	24.3 (18.1)		25.3 (19.2)
10^6	59.0 (19.5)		69.0 (9.3)	55.7 (12.9)		45.7 (23.9)
10^7	94.3 (1.9)		96.7 (0.2)	92.7 (1.5)		68.3 (15.2)
F (df)		0.28 (1, 12)			1.86 (1, 12)	
P		0.60			0.20	
	GHA	*t*	17-41	GHA	*t*	17-41
10^4	16.7 (8.7)		26.7 (12.7)	8.3 (4.2)		25.0 (7.6)
10^5	31.8 (9.9)		32.6 (14.1)	18.3 (8.2)		32.5 (9.2)
10^6	60.7 (23.2)		55.4 (20.7)	59.0 (19.4)		55.4 (23.8)
10^7	92.8 (1.5)		96.7 (0.2)	89.4 (0.7)		90.0 (0.5)
F (df)		0.01 (1, 12)			0.79 (1, 12)	
P		0.94			0.39	
	GHA	*t*	44-03	GHA	*t*	44-03
10^4	13.0 (11.9)	1.0	2.8 (7.3)	0.00 (0.00)	0.64	6.5 (1.4)
10^5	5.3 (3.3)	1.71	22.8 (7.3)	9.8 (0.04)	1.47	24.3 (5.4)
10^6	26.0 (4.8)	4.38*	63.8 (13.7)	28.5 (5.0)	3.15*	53.8 (32.7)
10^7	87.3 (3.3)	2.34*	96.3 (0.6)	81.8 (0.9)	2.98*	93.8 (0.7)
F (df)		13.76 (1, 18)			19.98 (1, 18)	
P		0.0016			0.0006	
	Average percentage mortality (SE) (nymphs)			Average percentage sporulation (SE) (nymphs)		
	GHA	*t*	44-03	GHA	*t*	44-03
10^4	0.0 (0.0)	0.13	13.3 (2.7)	3.3 (0.7)		3.3 (0.7)
10^5	20.0 (6.0)	0.14	21.3 (9.7)	20.0 (0.0)		17.7 (3.5)
10^6	32.0 (4.9)	4.79*	74.0 (0.7)	30.7 (5.6)		64.7 (6.9)
10^7	81.3 (0.9)	2.69*	92.0 (0.9)	79.0 (2.7)		83.0 (0.5)
F (df)		19.74 (1, 12)			2.89 (1, 12)	
P		0.0008			0.11	

Analysis of variance was calculated with arcsine transformed percentage mortality (after correcting for control mortality) and arcsine transformed percentage sporulation. Means (SE) in table are not transformed.
t statistic not calculated if *F* NS.
* Significant difference between isolates at the given dose.

respect to the potential for horizontal transmission (Table 4). For example, if an isolate was effective at killing adults, but did not produce conidia, it may be less effective in the field at sustaining high levels of infection. SC_{50}s for percentage sporulation ranged from a low of 3.8×10^3 conidia/ml (isolate 35-26) to a high of 9.9×10^5 conidia/ml (isolate 47-41). However, it is interesting to note that isolate 47-41 actually had a lower LC_{50} for percentage mortality (1.4×10^4) than did isolate 35-26 (2.7×10^4) suggesting the possibility of toxin production in the former (i.e., a quick death occurred that did not allow total colonization of the fungus). These data should be evaluated with caution however, especially in relation to predicting horizontal transmission in the field. Laboratory studies provide ideal conditions for sporulation to occur. Field conditions, especially in hot dry areas where *Lygus* is a significant pest, may not support sporulation, thus minimizing the potential for horizontal transmission.

The probit model did not fit the data acquired from the repeated bioassays with three selected isolates (17-41, 44-03, and 54-43) well, due to very low and very high responses at low and high dose respectively and relatively high heterogeneity. Robertson and Preisler (1993) suggest that a different analysis be used for these types of data so the results were analyzed with analysis of variance and then each of the three experimental isolates were compared with GHA. Results suggest that only isolate 44-03 was significantly more effective at killing *L. hesperus* than GHA at 10^6 and 10^7 conidia/ml (Table 5). Similarly percentage sporulation was also significantly higher with 44-03. No significant differences were observed between isolates in percentage of the nymphs sporulating but results for percentage mortality were similar to those obtained from the adult bioassays in that 44-03 was significantly more efficacious at the higher two doses than GHA.

3.3. Genetic characterization

Seven microsatellite (SSR) markers were used to characterize 81 putative *B. bassiana* isolates recovered

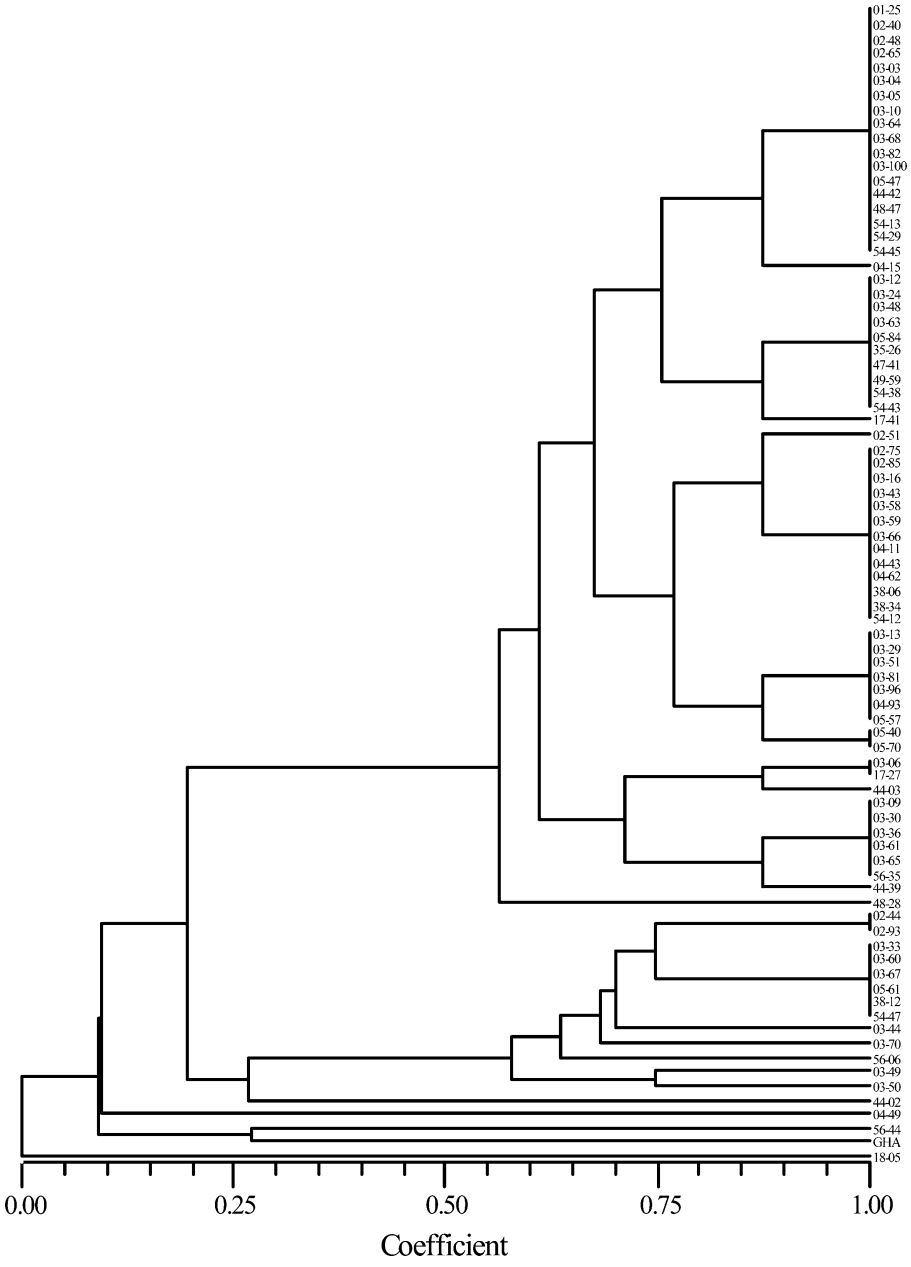

Fig. 1. Dendrogram, based on microsatellite markers, of genetic relatedness of *B. bassiana* isolates from *L. hesperus* from the San Joaquin Valley, CA and GHA. See Table 1 for isolate information and text for molecular biology procedures.

from California *L. hesperus* populations and GHA (Fig. 1). SSR markers yielded 44 amplicons from the isolates tested. SSR markers labeled with red well dye yielded from three (Ba06) to nine (Ba03) alleles (Table 2). Rehner and Buckley (2003), looking at much wider geographic populations of *B. bassiana* observed 10 alleles from Ba02. The widest size range observed in the present study was 199–284 bp from Ba08 whereas the widest size range observed by Rehner and Buckley (2003) was 110–175 bp from Ba05. However, the narrowest size range observed from both studies was from Ba06. Allele size ranged from 84 to 284 bp. Most importantly, the markers can be used to distinguish selected isolates from each other. For example, isolates 44-03 and 17-41 (Table 4) do not overlap with any other isolate and are very different from GHA. Interestingly, isolates 54-43 from Kern County, CA, and 35-26 from Madera County were identical and isolate 17-41 from Tulare County was closely related. Isolate 44-03 from Kern County was more distantly related to the other three isolates. The commercial isolate was very distantly related to all but one new isolate from Madera County. There did not seem to be any clear relationship among isolates recovered in various years, fields, and/or geographical location. Although based on a limited amount of data, the dendrogram, based on

genetic similarities (UPGMA and N-J) of 81 CA isolates of *B. bassiana* suggests the existence of at least four clades all fairly closely related suggesting a relatively close relationship among isolates from the same geographical locality and host. Genetic similarities among all isolates ranged from 0.66 to 1.0. This phenomenon was also observed by Wang et al. (2003) who, in a study of 56 isolates using three molecular methods, concluded that genetic differences among isolates of *B. bassiana* were less among isolates from the same geographic locality than from different hosts.

In summary, at least one isolate (44-03) of *B. bassiana* recovered from *L. hesperus* in California appears to have attributes that suggest better adaptation to the local environment and host. However, as many authors point out (e.g., Fargues and Remaudiere, 1977; Liu et al., 2002, 2003; Sun et al., 2003; Yeo et al., 2003), there are many factors that must be considered before development of any isolate as a potential microbial pesticide proceeds. Several studies with collaborators are ongoing to enable selection of the best isolates for further study. Only through exhaustive studies, involving the efforts of many, does any isolate have a chance at becoming a functional microbial pesticide.

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